

WHAT IS CLAIMED IS:

1. A base type determination method for determining  
a base type of a monobasic substituted region of a target nucleic  
5 acid, the method comprising the steps of:

(a) preparing a solution containing a target  
double-stranded nucleic acid having the monobasic substituted  
region, a base type determination primer, a DNA polymerase, and  
dNTPs;

10 (b) causing the base type determination primer to  
hybridize to the target double-stranded nucleic acid in the  
solution, and causing a primer extension reaction to start  
progressing from the base type determination primer; and

(c) analyzing the degree of progress of the primer  
15 extension reaction to determine the base type of the substituted  
region,

wherein the base type determination primer consists of  
a first single-stranded nucleic acid which is capable of, when  
hybridizing to the target double-stranded nucleic acid,  
20 hybridizing to one of two strands of the target double-stranded  
nucleic acid such that a 3' terminal of the primer corresponds  
to the substituted region of said one strand of the target  
double-stranded nucleic acid, and

wherein the first single-stranded nucleic acid consists  
25 of:

a substitution corresponding region which is located at the 3' terminal and consists of one base complementary to any one of predictable types of bases in the substituted region of said one strand of the target double-stranded nucleic acid;

5            an uncomplementary region which is located adjacent to the substitution corresponding region on a 5' terminal side thereof and consists of two bases uncomplementary to said one strand of the target double-stranded nucleic acid; and

          a complementary region which is located adjacent to  
10 the uncomplementary region on the 5' terminal side thereof and consists of five or more bases complementary to said one strand of the target double-stranded nucleic acid.

2. The base type determination method according to  
15 claim 1, wherein the DNA polymerase has substantially no 3'→5' exonuclease activity.

3. The base type determination method according to claim 1, wherein the first single-stranded nucleic acid is a DNA.

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4. The base type determination method according to claim 1,

          wherein in the step (a), the solution further contains a reverse primer consisting of a single-stranded nucleic acid  
25 capable of hybridizing to the other strand of the target

double-stranded nucleic acid, and

wherein in the step (b), the primer extension reaction is caused to progress using a base sequence amplifying method selected from the group consisting of a PCR, an SDA, an RCR, an  
5 LAMP, and a TMA.

5. The base type determination method according to claim 4, wherein in the step (c), the base type of the substituted region is determined based on a difference in progress of the primer  
10 extension reaction.

6. The base type determination method according to claim 4, wherein in the step (c), the degree of progress of the primer extension reaction is analyzed by using a method selected  
15 from the group consisting of electrophoresis, mass analysis, and liquid chromatography to measure the amount of amplification of a base sequence amplified by the base sequence amplifying method.

7. The base type determination method according to  
20 claim 1, wherein in the step (c), the degree of progress of the primer extension reaction is analyzed by measuring the amount of pyrophosphoric acid generated by the primer extension reaction.

8. The base type determination method according to  
25 claim 4, wherein in the step (c), the amount of amplification of

a base sequence amplified by the base sequence amplifying method is measured by measuring the amount of pyrophosphoric acid generated by the primer extension reaction.

5           9. The base type determination method according to claim 4, wherein in the step (c), the amount of amplification of a base sequence amplified by the base sequence amplifying method is quantitatively analyzed to determine the base type of the substituted region.

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          10. The base type determination method according to claim 7,

          wherein measurement of the amount of pyrophosphoric acid includes the steps of:

15           converting the pyrophosphoric acid into an inorganic phosphoric acid within a sample containing at least a portion of the solution resulted from the step (b);

          providing the sample to a measurement system including glyceraldehyde 3-phosphate, oxidized nicotinamide  
20   adenine dinucleotide, glyceraldehyde 3-phosphatedehydrogenase, and at least one electron-transfer mediator; and

          measuring a value of current generated in the measurement system, and

          wherein the value of current indicates a concentration  
25   of the pyrophosphoric acid in the sample.

11. The base type determination method according to claim 10, wherein said at least one electron-transfer mediator is selected from the group consisting of ferricyanide, 1,2-naphthoquinone-4-sulfonic acid, 2,6-dichlorophenol-indophenol, dimethylbenzoquinone, 1-methoxy-5-methylphenazinium sulfate, methylene blue, gallocyanine, thionine, phenazine methosulfate, and meldora blue.

12. The base type determination method according to claim 10, wherein the measurement system further include diaphorase.

13. The base type determination method according to claim 10, wherein the pyrophosphoric acid is converted into the inorganic phosphoric acid by causing the pyrophosphoric acid to react with pyrophosphatase in the sample.

14. The base type determination method according to claim 7,

wherein measurement of the amount of the pyrophosphoric acid includes the steps of:

placing a sample including at least a portion of a solution resulted from the step (b) in one region of a measurement system having at least two regions divided by a membrane which

holds  $H^+$ -pyrophosphatase and has a limited permeability to  $H^+$ ;  
and

measuring a change in concentration of  $H^+$  in either  
one of said at least two regions of the measurement system, and

5            wherein the degree of the change in concentration of  
 $H^+$  indicates the concentration of the pyrophosphoric acid in the  
sample.

15            15. The base type determination method according to  
claim 14,

wherein the measurement of the pyrophosphoric acid  
includes the steps of:

providing the sample including at least a portion  
of a solution resulted from the step (b) to a measurement system  
15 including an artificial or natural membrane vesicle containing  
 $H^+$ -pyrophosphatase therein; and

measuring the change in concentration of  $H^+$  in the  
inside or outside of the membrane vesicle, and

20            wherein the degree of the change in concentration of  
 $H^+$  indicates the concentration of the pyrophosphoric acid in the  
sample.

16. The base type determination method according to  
claim 14, wherein the change in concentration of  $H^+$  is measured  
25 by either a method which measures an optical change converted from

the change in concentration of  $H^+$  or a method which measures an electrical change converted from the change in concentration of  $H^+$ .

5                   17. The base type determination method according to claim 16, wherein the method which measures an optical change uses a pH test paper, a pH-sensitive dye, or a membrane potential-sensitive dye.

10                   18. The base type determination method according to claim 16, wherein the method which measures an electrical change is selected from the group consisting of a metal electrode method, a glass electrode method, an ISFET electrode method, a patch-clamp method, and an LAPS method.

15                   19. The base type determination method according to claim 17, wherein the method which measures an optical change uses the pH-sensitive dye to measure the change in concentration of  $H^+$  in the inside of the membrane vesicle.

20                   20. The base type determination method according to claim 1,

                  wherein in the step (a), the solution further contains a second base type determination primer,

25                   wherein the second base type determination primer

consists of a second single-stranded nucleic acid capable of, when hybridizing to the target double-stranded nucleic acid, hybridizing to one of two strand of the target double-stranded nucleic acid which is the same strand as that to which the first base type determination primer is supposed to hybridize, such that a 3' terminal of the second base type determination primer corresponds to the substituted region of said one strand, and wherein the second single-stranded nucleic acid includes:

10 a second substitution corresponding region located at the 3' terminal and consisting of one base which is complementary to any one of predictable types of bases in the substituted region of the target double-stranded nucleic acid and is different in type from said one base of the substitution corresponding region  
15 of the first single-stranded nucleic acid;

a second uncomplementary region which is adjacent to the second substitution corresponding region on the 5' terminal side and consists of two bases uncomplementary to said one strand of the target double-stranded nucleic acid; and

20 a second complementary region which is adjacent to the second uncomplementary region on the 5' terminal side and consists of five or more bases complementary to said one strand of the target double-stranded nucleic acid.

25 21. The base type determination method according to



claim 20, wherein the first single-stranded nucleic acid and the second single-stranded nucleic acid are different in length from each other.

5           22. The base type determination method according to claim 20, wherein the first single-stranded nucleic acid and the second single-stranded nucleic acid are labeled by their respective fluorescences which are different in wavelength.

10           23. A base type determination primer for determining a base type of a monobasic substituted region of a target nucleic acid,

          wherein the primer consists of a single-stranded nucleic acid which is capable of hybridizing to the target nucleic acid  
15 such that a 3' terminal of the primer corresponds to the substituted region of the target nucleic acid, and

          wherein the single-stranded nucleic acid includes:

          a substitution corresponding region which is located at the 3' terminal and consists of one base complementary to any  
20 one of predictable types of bases in the substituted region of the target nucleic acid;

          an uncomplementary region which is located adjacent to the substitution corresponding region on a 5' terminal side thereof and consists of two bases uncomplementary to the target  
25 nucleic acid; and

a complementary region which is located adjacent to the uncomplementary region on the 5' terminal side thereof and consists of five or more bases complementary to the target nucleic acid.

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24. A base type determination reagent kit for determining a base type of a monobasic substituted region of a target nucleic acid, the kit comprising a base type determination primer, a DNA polymerase, and dNTPs,

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wherein the primer consists of a first single-stranded nucleic acid which is capable of hybridizing to the target nucleic acid such that a 3' terminal of the primer corresponds to the substituted region of the target nucleic acid, and

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wherein the first single-stranded nucleic acid includes:

a substitution corresponding region which is located at the 3' terminal and consists of one base complementary to any one of predictable types of bases in the substituted region of the target nucleic acid;

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an uncomplementary region which is located adjacent to the substitution corresponding region on a 5' terminal side thereof and consists of two bases uncomplementary to the target nucleic acid; and

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a complementary region which is located adjacent to the uncomplementary region on the 5' terminal side thereof and

consists of five or more bases complementary to the target nucleic acid.

25. The base type determination reagent kit according  
5 to claim 24, wherein the DNA polymerase has substantially no 3'→5'  
exonuclease activity.

26. The base type determination reagent kit according  
to claim 24, wherein the first single-stranded nucleic acid is  
10 a DNA.

27. The base type determination reagent kit according  
to claim 24, further comprising a reverse primer.

15 28. The base type determination reagent kit according  
to claim 24, further comprising pyrophosphatase.

29. The base type determination reagent kit according  
to claim 28, further comprising glyceraldehyde 3-phosphate,  
20 oxidized nicotinamide adenine dinucleotide, glyceraldehyde  
3-phosphatedehydrogenase, and at least one electron-transfer  
mediator.

30. The base type determination reagent kit according  
25 to claim 29, further comprising diaphorase.

31. The base type determination reagent kit according to claim 29, wherein said at least one electron-transfer mediator is selected from the group consisting of ferricyanide, 1,2-naphthoquinone-4-sulfonic acid, 2,6-dichlorophenol-indophenol, dimethylbenzoquinone, 1-methoxy-5-methylphenazinium sulfate, methylene blue, gallocyanine, thionine, phenazine methosulfate, and meldora blue.

32. The base type determination reagent kit according to claim 24, further comprising  $H^+$ -pyrophosphatase.

33. The base type determination reagent kit according to claim 32, further comprising a pH test paper, a pH-sensitive dye, or a membrane potential-sensitive dye.

34. The base type determination reagent kit according to claim 24, further comprising a second base type determination primer,

wherein the second base type determination primer consists of a second single-stranded nucleic acid capable of hybridizing to the target nucleic acid such that the 3' terminal corresponds to the substituted region of the same strand as that to which the first base type determination primer is supposed to hybridize, and

wherein the second single-stranded nucleic acid includes:

a second substitution corresponding region located at the 3' terminal and consisting of one base which is complementary to any one of predictable types of bases in the substituted region of the target nucleic acid and is different in type from said one base of the substitution corresponding region of the first single-stranded nucleic acid;

a second uncomplementary region which is located adjacent to the second substitution corresponding region on a 5' terminal side thereof and consists of two bases uncomplementary to the target nucleic acid; and

a second complementary region which is located adjacent to the second uncomplementary region on the 5' terminal side thereof and consists of five or more bases complementary to the target nucleic acid.

35. The base type determination reagent kit according to claim 34, wherein the first single-stranded nucleic acid and the second single-stranded nucleic acid are different in length from each other.

36. The base type determination reagent kit according to claim 34, wherein the first single-stranded nucleic acid and the second single-stranded nucleic acid are labeled by their

respective fluorescences which are different in wavelength.